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Research Article

Biodegradation of Aged Residues of Atrazine and Alachlor in a Mix-Load Site Soil by Fungal Enzymes

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Soils from bulk pesticide mixing and loading (mix-load) sites are often contaminated with a complex mixture of pesticides, herbicides, and other organic compounds used in pesticide formulations that limits the success of remediation efforts. Therefore, there is a need to find remediation strategies that can successfully clean up these mix-load site soils. This paper examined the degradation of atrazine (2-chloro-4-ethylamino-6-isopropylamino-S-triazine; AT) and alachlor (2-chloro-2′, 6′-diethyl-N-[methoxymethyl]-acetanilide) in contaminated mix-load site soil utilizing an extracellular fungal enzyme solution derived from the white rot fungus, *Phanerochaete chrysosporium*, grown in a packed bed bioreactor. Thirty-two percent of AT and 54% of AL were transformed in the biometers. The pseudo first-order rate constant for AT and AL biodegradation was 0.0882 d^{-1} and 0.2504 d^{-1}, respectively. The half-life ($t_{1/2}$) for AT and AL was 8.0 and 3.0 days, respectively. Compared to AT, the initial disappearance of AL proceeded at a faster rate and resulted in a greater amount of AL transformed. Based on the net Co_2 evolved from the biometers, about 4% of the AT and AL initially present in the soil was completely mineralized.

1. Introduction

Bulk pesticide mixing and loading (mix-load) sites are major contributors of pesticide contamination of ground and surface waters [1]. Many of these sites contain extremely high concentrations of pesticides, fertilizers, and other organic compounds used in pesticide formulations, which may limit bioremediation efforts due to the toxicity effects of the high pesticide concentrations to the indigenous microbes [2, 3], the complexity of the mixture of compounds present [4], and the natural heterogeneity of the soil and water environment [5]. Remediation treatment strategies must evaluate and overcome the difficulties associated with mix-load sites.

Bioaugmentation has become a cost-effective alternative for cleanup of contaminated soil and groundwater. Although bacterial degradation schemes are utilized more often, there is great potential for the use of fungal degradative systems. Certain fungi, particularly the white rot fungi (WRF), are often more successful degrading pesticides than other microorganisms because of their ability to tolerate and/or detoxify pesticides found in complex mixtures and at high concentrations, such as those soils from mix-load sites [4].

The WRF, *Phanerochaete chrysosporium*, has been shown to degrade a variety of pesticides in the laboratory. However, field applications have not had as much success due to the difficulties with growing the fungi to sufficient biomass for soil application. The complex interactions among fungal inoculant, soil type, and soil microflora have led to failure of field scale experiments [6, 7]. Several studies have seen competition by indigenous organisms during degradation while other studies have isolated microbes from the soil that are antagonistic to *P. chrysosporium* [4, 7].

The ligninolytic growth phase of WRF produces peroxidases, lactases, laccases, hydrogen peroxide-generating systems, other enzymes, and cofactors [4, 6, 8] that are capable of degrading recalcitrant compounds. Because of the extracellular nature of these ligninolytic enzymes, researchers investigated the potential of utilizing the fungal enzyme solution directly in the contaminated soil. Rodríguez-Couto et al. [9] successfully decolorized the polymeric dye, Roly R-478,

in aqueous cultures using the extracellular culture fluid that contained primarily LiP from a semisolid state bioreactor of *P. chrysosporium*. In another study with *P. chrysosporium*, ligninolytic enzymes separated from mycelia were able to continue bleaching hardwood kraft pulp for 5 days [10]. These successes have confirmed that the extracellu-lar enzymes of the WRF can be active in soil.

A contaminated soil from a 100-year-old mix-load site located in Reading, Pa was found to contain high concentrations of pesticides and fertilizers [11]. Soil concentrations of atrazine (2-chloro-4-ethylamino-6-isopropylamino-S-triazine; AT) and alachlor (2-chloro-2′,6′-diethyl-N-[methoxymethyl]-acetanilide; AL) were above the EPA risk-based concentrations (RBC) for commercial areas [12], with 52 mg kg^{-1} AT (RBC = 13.0 mg kg^{-1}) and 200 mg kg^{-1} AL (RBC = 36.0 mg kg^{-1}). The purpose of this paper was to develop a feasible bioremediation technique for the contaminated mix-load site soil utilizing the unique ligninolytic system of the WRF. Specifically, the objective of this research was to examine the degradation of AT and AL in both aqueous batch microcosms and in the contaminated mix-load site soil utilizing the fungal extracellular enzyme solution (EES) derived from the white rot fungus, *Phanerochaete chrysosporium*.

2. Materials and Methods

2.1. Enzyme Inoculum Preparation. Production of the ligninolytic enzymes was achieved by growing fungal cultures in a packed bed bioreactor (PBR) operated in semicontinuous mode based on the methods of Feijoo et al. [13]. *Phanerochaete chrysosporium* cultures (ATCC #34541) were maintained on 2% malt agar slants at ambient temperature. In preparation for spore production, the fungal cultures were transferred to petri dishes containing the 2% malt agar and incubated for two to five days at 39°C following the procedure of Tien and Kirk [14]. The harvested conidia (10 mL, A650 = 0.18) were utilized for mycelia production in shallow stationary cultures containing 75 mL of growth media. The flasks were maintained at 37°C and gently flushed with 100%, filter-sterilized (0.2 um) oxygen (98% Purity; 0.5 bars) for 5 minutes every day. After sufficient growth, the mycelia were harvested, homogenized, and then immobilized onto polyurethane foam cubes (1 cm^3, ρ = 354 kg m^{-3}) following the procedures of Feijoo et al. [13].

The jacketed PBR was heated by pumping water (37°C) through the column. Filter-sterilized (0.2 μm), high purity oxygen was purged (0.5 bar) through the column to maintain aerobic conditions. Flow was produced by peristaltic pumps (Model 77120-30) with an influent flow rate of 0.4 mL min^{-1} and a recycle flow rate of 0.7 mL min^{-1} resulting in a hydraulic retention time of approximately 8 hours. The polyurethane foam cubes containing immobilized *P. chrysosporium* were aseptically transferred to the 200 mL working volume of the bioreactor. After sufficient growth was achieved in the PBR, the influent was switched to a nitrogen-limiting media to induce ligninolytic activity, and the overflow effluent was monitored for LiP and MnP production.

Lignin peroxidase activity was determined by measuring the oxidation of veratryl alcohol (VA) to veratraldehyde at 310 nm (ε = 9,300 M^{-1}cm^{-1}) [14]. The reaction mixture contained 0.2 mL 10 mM VA; 0.2 mL 0.25 M d-tartaric acid, pH 2.5; 0.08 mL H_2O_2 (30%); 0.520 mL of collected bioreactor effluent manganese-dependent peroxidase activity was determined by the oxidation of o-dianisidine (-2HCl) at 460 nm (ε = 29,400 M^{-1}cm^{-1}) [15]. The reaction mixture contained 0.1 mL 1 mM manganese sulfate; 0.2 mL 0.5 M sodium tartrate, pH 5.0; 0.1 mL 1 mM H_2O_2; 0.1 mL 1 mM o-dianisidine; 0.500 mL of collected bioreactor effluent. Activity was expressed in U l^{-1}, where 1 U = 1 μmol min^{-1}. The extracellular enzyme solution (EES) used for the degradation studies contained 149.0 U l^{-1} LiP and 77.6 U l^{-1} MnP.

2.2. AT and AL Biodegradation in Aqueous Cultures. Standard aqueous cultures containing selective media (AT and AL as the only source of carbon and nitrogen) were utilized to evaluate the catalytic activity of the EES. The microcosms consisted of 250 mL Erlenmeyer flasks containing 50 mL of the selective herbicide solution with 27.0 mg L^{-1} AT, 21.16 mg L^{-1} AL, 0.5 mL concentrated phosphate buffer (pH 6.8), 0.5 mL concentrated salt solution, and 0.5 mL trace elements [1]. The following three treatments were evaluated: (1) 5 mL of the collected enzyme solution, (2) 5 mL of the collected enzyme solution and 1 mL 5 mM H_2O_2, and (3) 5 mL of sterilized distilled deionized water (DDW) and 1 mL 5 mM H_2O_2 as the control. The flasks were sampled on days 0, 1, 2, 5, 7, 9, and 10. Prior to sampling the flasks were weighed and corrected for evaporative water loss by addition of sterile water. A 0.5 mL aliquot was removed and mixed with 0.5 mL acetonitrile and centrifuged at approximately 12,000 × g for 10 minutes. The supernatant was transferred to HPLC vials for analysis.

2.3. AT and AL Biodegradation in Soil Microcosms. Aerobic microcosms were prepared in standard 250 mL biometer flasks (Bellco, Glass Inc.,Vineland, NJ) consisting of a main body with a side-arm trap containing 0.2 N NaOH prepared with CO_2-free DDW. Fifty grams of air-dried, ground soil collected from a 100-year-old mix-load site located in Reading, Pa [1] were placed into the main body of the biometer flasks. Site 5A soil was chosen for this study because of the lack of AT and AL degraders [1] and the presence of high concentrations AT and AL (Table 2). The flasks were autoclaved at 120°C (290 bar) on three consecutive days before treatment. Three control flasks received 5 mL of sterile DDW and 1 mL 5 mM H_2O_2. The 3 treatment flasks received 5 mL of the EES collected from the PBR as described previously. Before addition to the biometers, the treatment solution (EES or DDW) and the H_2O_2 were mixed well for 30 sec and then added to the flasks. Biometer flasks were incubated at 23°C for the length of the experiment. The biometers were monitored for AT, AL, and metabolites. The entire experiment was done in triplicate.

The biometer flasks were monitored for CO_2 and herbicide concentration on days 1, 2, 3, 4, 7, and 13. The NaOH in the biometer side-arm traps was extracted and analyzed

Table 1: A list of the compounds monitored by HPLC and % recovery at the 95% level of confidence. All compounds were purchased from ChemService, Inc. (West Chester, Pa) and used as received.

	Label	% Recovery
Atrazine	CIET	100.5 ± 5.8
Atrazine deethyl	CIAT	38.2 ± 3.2
Atrazine deethyl-2-hydroxy	OIAT	8.04 ± 2.4
Atrazine-2-hydroxy	OIET	8.00 ± 1.7
Atrazine deethyl deisopropyl-2-hydroxy	OAAT	23.8 ± 7.9
Atrazine deethyl deisopropyl	CAAT	24.8 ± 6.4
Atrazine deisopropyl	CEAT	16.3 ± 1.8
Alachlor	AL	97.1 ± 8.7
2-chloro-2′,6′-diethylacetamilide	DMA	100.6 ± 9.2
2,6-diethylanaline	DIE	50.1 ± 7.4
Cyanazine	CY	87.6 ± 5.4
Metolachlor	ME	84.6 ± 6.5
Simazine	SI	86.4 ± 7.6
Simazine hydroxy	SIOH	
Aniline	ANI	44.02 ± 11

Table 2: Physical and chemical properties of Site 5A soil (dry wt. basis).

Sample	S-5A
Location	East side of loading dock
Textural class	loamy sand
SOM %	1.1 (0)[a]
pH	7.33
NH_4-N (mg kg^{-1})	10.49 (0.31)
NO_3-N (mg kg^{-1})	50.24 (3.12)
Sol Salts (mmho cm^{-1})	1.34 (0.11)
Acidity (meq 100 g^{-1})	0.033 (0.029)
Moisture content, field capacity (%)	8.61
Atrazine (mg kg^{-1})	205.1 (10.2)
Alachlor (mg kg^{-1})	108.5 (8.3)
Cyanazine (mg kg^{-1})	2272.3 (145.5)
Simazine (mg kg^{-1})	13.15 (1.94)
Metolachlor (mg kg^{-1})	1829.4 (106.4)

[a] Number in parentheses represents standard deviation of 3 values.

for CO_2 by the traditional acid titration method outlined by Zibilske [16]. Approximately 1 g aliquots of soil were removed from the flasks for herbicide determination.

2.4. Herbicide Analysis. The soils were analyzed for AT, AL, and metabolites using a solid-phase extraction (SPE) method followed by HPLC analysis [17]. The solid-phase extraction method was modified in order to extract possible polar metabolites. The 1 g soil aliquots were extracted with 50 mL 90% methanol and centrifuged (2400 g × rpm) for 20 minutes. A 5 mL aliquot of the supernatant was diluted with 55 mL DDW before SPE. Solid-phase extraction of the diluted aliquots was performed following the method of Chirnside et al. [11]. The resulting elutant was evaporated to dryness, redissolved with 5 mL acetonitrile, and transferred to HPLC vials for analysis.

A 20 μL aliquot of the prepared samples was analyzed with a Thermo Separations Product-AS3000 Series HPLC equipped with a UV photodiode array detector set at 220 nm and the reversed phase Phenomenex Synergi 4 μ Polar-RP column (150 × 4.6 mm, dp = 4 μm) set at ambient temperature. A gradient mobile phase consisting of (a) acetonitrile and (b) 0.003 M potassium dihydrogen phosphate, pH = 3.00 was used. Percent recoveries of AT, AL, and metabolites were determined by replicate analysis of reference soil spikes (Table 1).

3. Results

3.1. AT and AL Biodegradation in Aqueous Cultures. There was an immediate decrease in both AT and AL concentration for both treatments (Figure 1). There was about a 10- to-15-minute period between the two tasks of adding the EES to the flasks and removing the aliquot for analysis. The enzymatic reactions progressed immediately within the treatment flasks. The initial AT and AL concentration in all flasks was approximately 29 ± 1.21 mg L^{-1} (0.1351 mM) and 189 ± 2.96 mg L^{-1} (0.6995 mM), respectively. Both of the treatments showed a further decrease in AT and AL concentration during the test period. Treatment 2 (enzyme plus hydrogen peroxide) resulted in the greatest decrease in concentration with 14.4% of the AT (0.0983 mM, d 10) and 15.9% of the AL (0.5880 mM, d 10) transformed. Treatment 1 resulted in a 12.1% (0.1188 mM, d 10) and 11.4% (0.6195 mM at d 10) reduction of AT and AL, respectively.

Only very low levels of atrazine metabolites were produced in Treatment 1 flasks (Figure 2). The deisopropylatrazine (CEAT) metabolite was detected on day 0 but

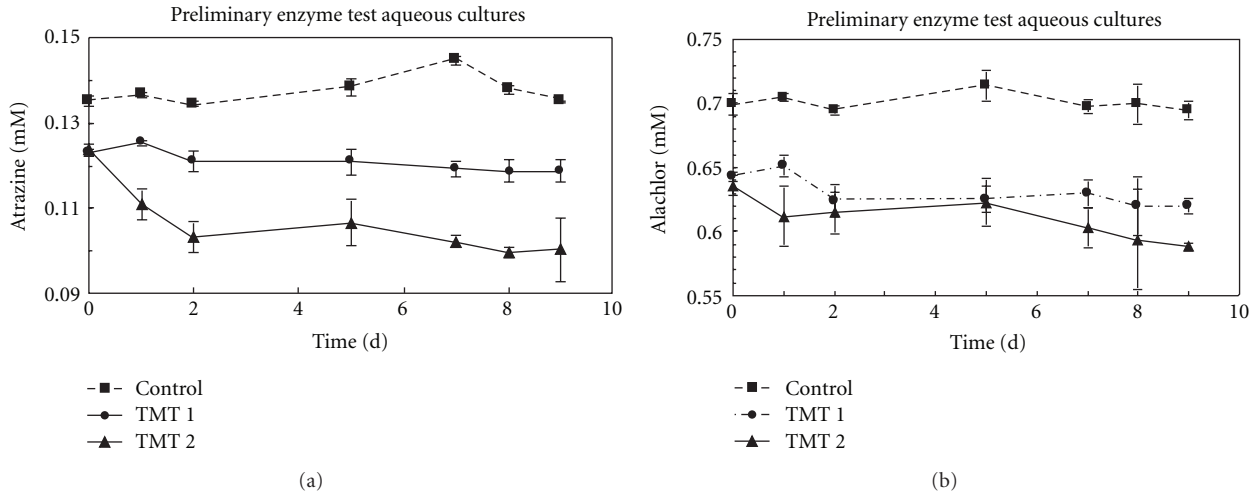

Figure 1: Disappearance of AT and AL from aqueous cultures treated with the EES. Treatment 1 consisted of the addition of the EES only, while Treatment 2 consisted of the addition of the EES and H_2O_2. (a) AT degradation among treatments. (b) AL degradation among treatments. Control consisted of DDW and H_2O_2.

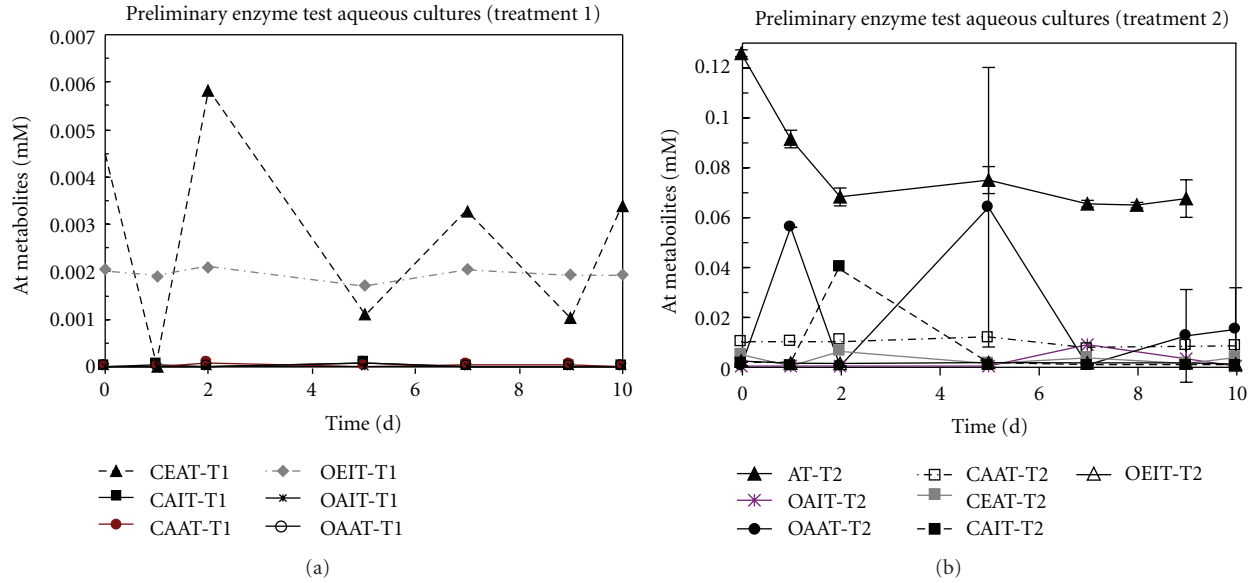

Figure 2: Production of atrazine metabolites during preliminary enzyme test in aqueous cultures. (a) Treatment 1 (EES only). (b) Treatment 2 (EES and H_2O_2). OIET = atrazine-2-hydroxy, CAAT = atrazine deethyl deisopropyl, OAAT = atrazine deethyl deisopropyl-2-hydroxy, CEAT = atrazine deisopropyl, OIAT = atrazine deethyl-2-hydroxy.

was completely transformed the following day. The level increased again and was further transformed throughout the test period. Hydroxyatrazine (OEIT) was seen on day 0 but the concentration remained the same throughout the incubation. In Treatment 2, a low level of the bis-dealkylated metabolite (CAAT) was seen on day 0 and remained constant throughout the incubation period. The dechlorinated bis-dealkylated metabolite (OAAT) was detected on day 1. It was further degraded, but more OAAT was seen on day 5. Again, the OAAT was further degraded with very little remaining at the end of the incubation period. As in Treatment 1, the double dealkylated metabolite (CAAT) was present at a constant low concentration throughout the experiment.

Figure 3 illustrates the production of the two alachlor metabolites, DMA and DIE, for both Treatment 1 and Treatment 2. Both treatments produced very low concentrations of DMA. The aniline metabolite (DIE) was only present at low concentrations in Treatment 2.

3.2. AT and AL Biodegradation in Soil Microcosms

3.2.1. CO_2 Evolution Rates. A steady increase in CO_2 evolution was seen for both treatments (Figure 4). However, the CO_2 evolution in the treated biometers increased at a rate approximately two times greater than the background CO_2 of the untreated biometers (0.0400 mM CO_2 d^{-1} versus

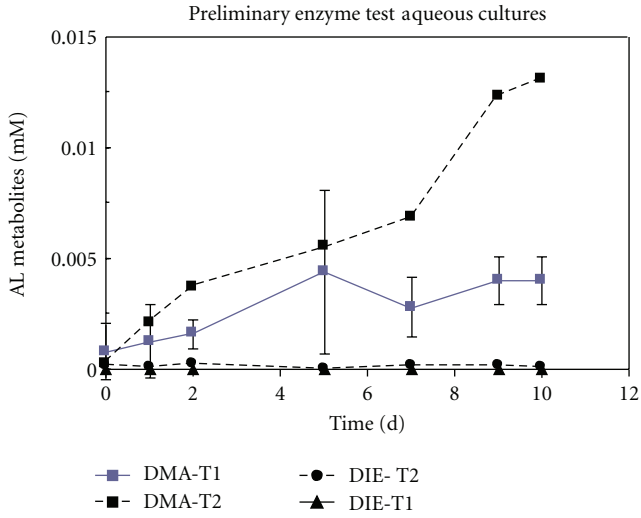

Figure 3: Production of alachlor metabolites during preliminary enzyme test in aqueous cultures. Treatment 1 consisted of the EES only and Treatment 2 consisted of the EES and H_2O_2. DMA = 2-chloro-2′,6′-diethylacetamilide, DIE = 2,6-diethylanaline.

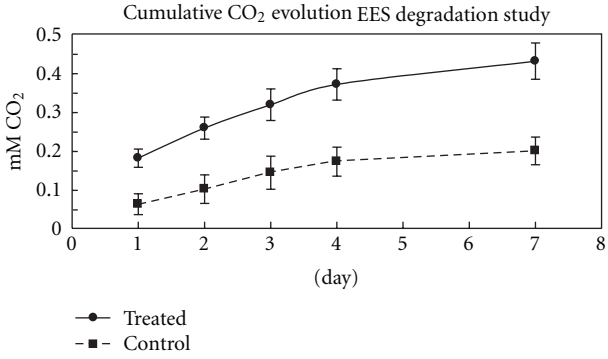

Figure 4: Evolution of CO_2 during the incubation period of the soil degradation study, which assessed the degradative ability of the EES applied to pesticide-contaminated soil. Cumulative evolution of CO_2 in treated and control biometers. Values are the average of 9 samples. Error bars represent 1 standard deviation.

0.0223 mM CO_2 d^{-1}). The total amount of CO_2 evolved from the treatment biometers was significantly different from the control biometers according to the Kruskal-Wallis one-way analysis of variance on ranks ($P < 0.001$). The rate of CO_2 produced during the first 3 days of incubation was approximately two times greater than the overall evolution rate (0.0687 mM d^{-1}).

3.2.2. Herbicide Degradation . During the incubation period, atrazine concentration decreased from an initial concentration of 0.730 ± 0.091 mM to a final concentration of 0.4891 ± 0.011 mM. A 15% decrease in AT concentration was seen by the first day after treatment with the EES. As seen in Figure 5(a), AT degradation continued for another day at which time no additional AT was degraded. No significant degradation was seen in the control biometer flasks. Assuming a pseudo first-order reaction for the disappearance of AT, the plot of the natural logarithm of initialized AT concentration (C/C_o) versus time yielded the first-order rate constant of 0.0882 d^{-1}. The half-life ($t_{1/2}$) for AT calculated from the plot was 8.0 days.

During the incubation period, alachlor concentration decreased from an initial concentration of 0.640 ± 0.125 mM to a final concentration of 0.297 ± 0.075 mM. Compared to AT, the initial disappearance of AL proceeded at a faster rate and resulted in a greater amount of AL transformed. A 35% decrease in AL concentration was seen by the first day after treatment with the EES. As seen in Figure 5(b), AL degradation continued for another day at which point no additional AL was degraded. No significant degradation was seen in the control biometer flasks. Assuming a pseudo first-order reaction for the disappearance of AL, the plot of the natural logarithm of initialized AL concentration (C/C_o) versus time yielded the first-order rate constant of 0.2504 d^{-1}. The half-life ($t_{1/2}$) for AL calculated from the plot was 3.0 days.

There were no significant changes in metabolite concentrations within the control biometer flasks. All of the monitored AT metabolites except atrazine deethyl-2-hydroxy were seen in the treated biometer flasks (Figure 6(a)). Within 24 hours, all metabolites detected exhibited an increase in concentration except for hydroxyatrazine (OEIT; Figure 6(b)). The most extensive changes occurred with the dealkylated metabolites. Deethylatrazine (CAIT) exhibited the greatest increase on day one and then slowly decreased in concentration until day 3, at which point no further change was seen. The bis-dealkylated metabolite (CAAT) and deisopropylatrazine (CEAT) showed a similar change in concentration increasing until day 3. The double dealkylated metabolite increased once again before the concentration slowly decreased whereas CEAT exhibited a decrease in concentration from day 3 until the end of the incubation period.

Alachlor degradation resulted in the formation of the 2-chloro-2′,6′-diethylacetanilide (DMA) and 2,6-diethylaniline (DIE) (Figure 7). Initially there was an increase in DMA but by day 2 the concentration began to decrease. A slight increase was seen at day 4 at which point no further change was seen. There was a slow increase in DIE until day 2 and then the concentration gradually decrease until day 4 at which point the concentration remained the same. The change in the DIE metabolite was minimal throughout the incubation period.

4. Discussion

4.1. AT and AL Biodegradation in Aqueous Cultures. In both enzyme treatments there was an immediate decrease in the concentration of both AT and AL. The amount of decrease was approximately equal for both treatments. Within 24 hr, however, the treatment with both the EES and H_2O_2 exhibited a greater decrease in AT and AL. The H_2O_2 activated the ligninolytic enzymes resulting in a greater production of hydroxy radicals available for further oxidations.

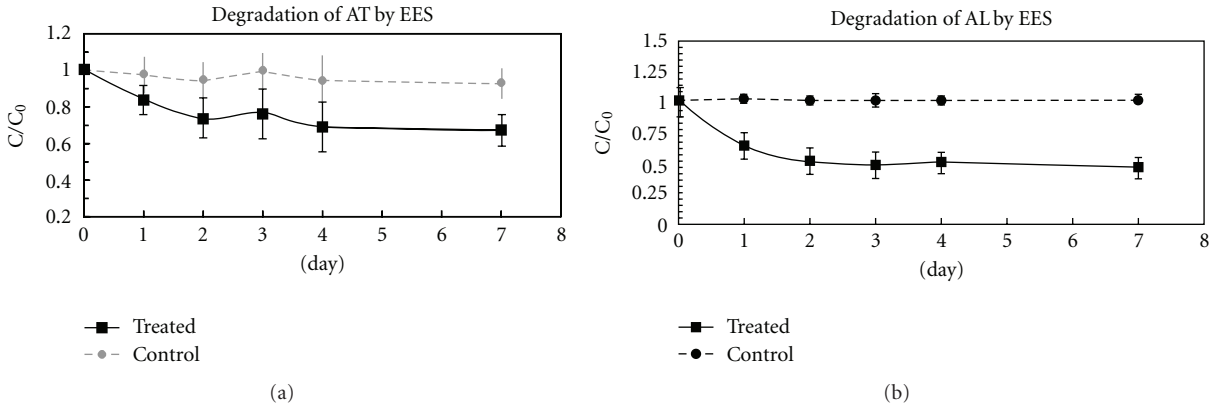

Figure 5: Disappearance of AT and AL over time from biometers of the EES degradation study. Data are the means of triplicate experiments. Error bars represent ± 1 standard deviation. (a) Atrazine concentration (C) is initialized and expressed as a fraction of the initial concentration (C_0). (b) Alachlor concentration (C) is initialized and expressed as a fraction of the initial concentration (C_0).

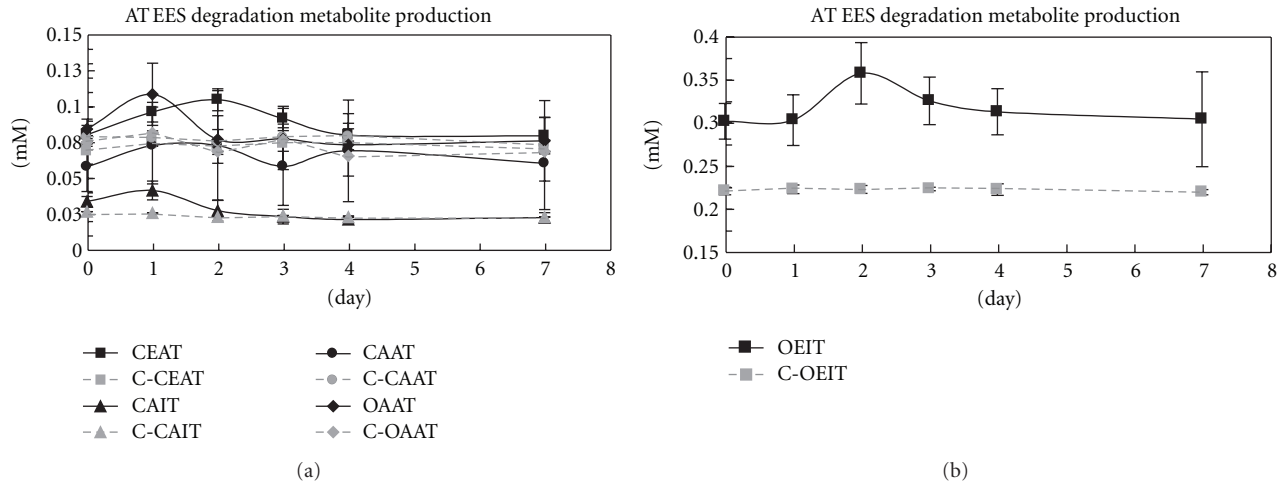

Figure 6: Production of atrazine metabolites over time in the EES degradation study. Metabolites found in control biometers (C-metabolite) are indicated by corresponding dotted lines. (a) All metabolites detected except for OEIT. (b) Hydroxyatrazine detected in biometer flasks. Numbers are the average of 9 values. Error bars represent one standard deviation of triplicate experiments.

The degradation of AT in both treatments resulted in the production of the dealkylated metabolites. The EES alone produced primarily deisopropyl atrazine (CEAT). Addition of H_2O_2 increased the production of all metabolites and caused the transformation of the initial metabolites to the bis-dealkylated products (CAAT, OAAT). These results are similar to those of Arnold et al. [18] in which the major products detected were the chlorinated metabolites, CAAT and CEAT. Only 5.8% of the AT was transformed to the dechlorinated metabolites. In reaction mixtures with higher concentration of H_2O_2, the dealkylation reaction was favored producing less dechlorinated products. Sequential batch treatments resulted in further transformation of the dealkylated products to the dechlorinated forms. In this work, very few dechlorinated products were formed and the treatment without H_2O_2 had more hydroxyatrazine produced. In Arnold et al.'s work [18], oxidation of the alkyl side chains occurred as well.

Both of the AL metabolites were detected in the reaction flasks. There was a greater amount of 2-chloro-2′,6′-diethylacetamilide (DMA) formed in Treatment 2 (EES and H_2O_2). The 2,6-diethylaniline (DIE) metabolite was detected in Treatment 2 only. In general, the amount of metabolites detected was very low. This suggested that the 2 products analyzed for were further degraded and thus did not accumulate within the reaction flasks. In the treatment flasks, analysis of the culture solution detected several unidentified peaks that may be further degradation products of the AL metabolites, DMA and DIE.

4.2. AT and AL Biodegradation in Soil Microcosms. Treatment of the pesticide-contaminated mix-load site soil with the extracellular enzyme solution resulted in degradation of 32% and 54% of the AT and AL, respectively. However, complete removal of the metabolites from the soil was not accomplished (Table 3). Catabolic activity occurred steadily

throughout the incubation period, while the net CO_2 evolved (0.2308 mM) indicated that approximately 12.2% of the AT and 4.0% of the Al was completely mineralized. Other studies with the white rot fungi have shown mineralization of both AT and AL. In work investigating several white rot fungi, Ferrey et al. [19] found that the fungi were able to mineralize the aromatic carbon (C) of AL to CO_2. After 122 days, *P. chrysosporium* mineralized approximately 6% of the alachlor (C_0 = 18 mg l^{-1}). Transformation also resulted in partially degraded metabolites, 37% in the aqueous fraction and 25% associated with the wood solids. In another study, 48% of AT in aqueous cultures was transformed by *P. chrysosporium* [20]. In an experiment utilizing ^{14}C-labeled AT, approximately 24% of the initial radioactivity from the side chain (ethyl) carbons was transformed to CO_2 by day 16 of the incubation period (18% of the total AT initially present). Very little of the ring Cs were mineralized to CO_2. However, the previous research was conducted with the fungus and isolated compounds in cultures containing no soil. The results from this research suggested that interactions of the transformation products with the complex nature of the mix-load site soil reduced the amount of net CO_2 produced. The nature of the fungal enzyme system produced free radicals, which could become involved in coupling reactions within the soil resulting in precipitates and/or binding of the transformation products to soil organic matter. In the work by Mougin et al. [20], 30% of the transformation products were immobilized bound residues. Many substituted anilines, especially chlorinated moieties, have been shown to strongly bind to soil humic substances [4]. Therefore the probability that a portion of the AL metabolites formed in these experiments became bound to the soil was great. *Streptomyces* sp and *P. chrysosporium* were reported to transform AT in contaminated soil with 30% immobilized by production of bound residues. Therefore, a portion of the degraded AT might have become bound to the soil. The dechlorinated metabolite, OEIT, has been shown to bind to soil at a greater extent than AT itself. Thus, the disappearance of OEIT in the biometers might have been the result of bound residue formation.

Another enigma encountered in this research was the nature of the interaction of the EES with soil components (mineral and organic). Adsorption or immobilization of enzymes on mineral colloids has affected their stability positively by enhancing their activity or negatively by inhibiting the activity of the protein molecules [21]. Changes induced in the EES through reactions with soil components might have resulted in the production of more bound residues. Immobilization of laccase onto soil mediated the transformation of guaiacol, 1-naphthol, and 4-chloro-1-naphthol into oligomeric products by C-C and C-O coupling reactions [21].

The rate constant for AT and AL biodegradation was determined to be 0.0882 d^{-1} and 0.2504 d^{-1}, respectively. These rates of AT and AL disappearance are considerably faster than the degradation by a selected microbial consortium isolated from the contaminated mix-load site soil [11]. The ligninolytic enzyme kinetic system is different than other microbial enzymes in that enzyme-substrate complexes are

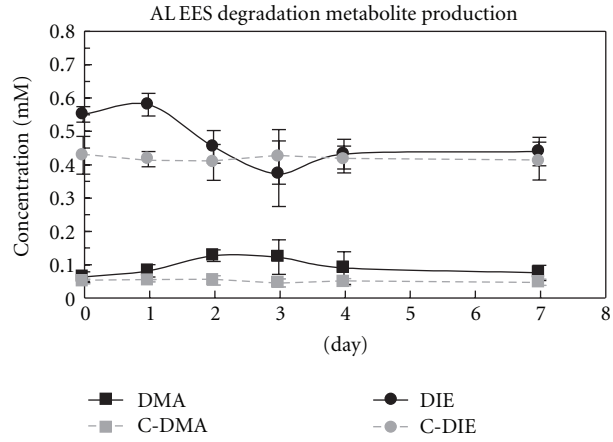

Figure 7: Production of alachlor metabolites over time in the EES degradation study. Numbers are the average of 9 values. Error bars represent one standard deviation of triplicate experiments.

not detected. Peroxidase activity is described as irreversible ping-pong kinetics; the enzyme and substrate must collide and form the covalently bound complex. The rate at which the complex reacts is fast compared to the rate in which it dissociates and thus reverts to the initial reactants. The result is that the rate of reaction appears to have no upper limit; that is, the greater the concentration of reactants the faster the reaction [22]. Thus, peroxidase enzymes exhibited a simpler steady-state rate equation. Overall, free radical mediated processes generally followed pseudo first-order kinetics [23]. These faster reaction rates were typical of the ligninolytic enzyme system.

Other rate data for reactions of peroxidases with AT and AL was limited in the literature, but comparisons were made to photolytic reactions. During indirect and direct photolysis of a substrate, the adsorption of UV-visible light energy resulted in the formation of oxidants such as the hydroxy radical (XOH) [24], causing similar reactions as described above. The observed rates constants calculated from first-order rate plots were 0.0960 d^{-1} and 0.6960 d^{-1} for direct and indirect photolysis. In the indirect process, the XOH are formed faster, thus the faster degradation rate [24]. This rate was similar to that calculated from the current research data, (0.0882 d^{-1} versus 0.0960 d^{-1}).

Direct photocatalysis (350 nm) of AL in aqueous solution resulted in a degradation rate of 7.14 d^{-1} [25]. Compared to this research work, the direct photocatalysis rate was exceedingly fast. However, the presence of soil constituents would have decreased degradation rates due to the scavenging of the free radicals produced by the EES. In the EES research work, the rate of degradation of AL was still almost 3 times as fast as the rate of AT disappearance. Considering the high oxidative state of the s-triazine ring, AL was a more attractive substrate for the oxidative reactions initiated by the EES. In fact, small methoxylated nonphenolic aromatics such as AL have served as crucial substrates for LiP [26]. Bollag et al. [27] found rapid dimerization of the AL metabolite, DIE, with the humic material, syringic acid. Thus, the disappearance

Table 3: Overview of AT and AL transformation and of the production and further transformation of metabolites within the EES-treated soil. (a) AT transformation. (b) AL transformation and the Net CO_2 evolved during incubation.

(a)

% AT removed	mM AT removed	
32.3	0.2359	
Metabolite	Formed (mM)	Degraded (mM)
CEAT	0.0242	0.0240
CAAT	0.0258	0.0234
OIET	0.0432	0.0089
OIAT	0.0	0.0
OAAT	0.0269	0.0355
CAIT	0.0089	0.0202
Total	0.1290	0.1120

(b)

% AL removed	mM AL removed	
53.5	0.3424	
Metabolite	Formed (mM)	Degraded (mM)
DMA	0.1252	0.0517
DIE	0.0957	0.2074
Total	0.2209	0.2591
Net CO_2 evolution (mM)		
0.2308		

of DIE in the biometers could have been the result of dimerization. Rapid transformation of AL in the EES-treated soil paralleled the processes outlined in the literature.

4.2.1. Metabolite Production and Degradation. For AT, the hydroxylated metabolites tended to accumulate in the biometer flasks without further transformation, while the dealkylated metabolites were further transformed. The increase in the dechlorinated N-dealkylated metabolites before the increase in hydroxyatrazine suggested that dechlorination of both atrazine and the double dealkylated metabolite occurred. There was a greater amount of deisopropyl atrazine (CEAT) present in the soil than deethyl atrazine (CAIT) indicating that reaction with the isopropyl side chain was favored over reaction with the ethyl side chain of AT.

In a study of AT degradation by the WRF, *Pleurotus pulmonarius*, in microcosms with spent mushroom substrate (SMS), both chlorinated and nonchlorinated metabolites were seen [28]. The monodealkylated chlorinated metabolites, CAIT and CEAT, were produced the fastest and reached higher concentrations during the first 2 weeks. They were quickly transformed and did not accumulate in the treated microcosms. The dechlorinated metabolites, especially OAAT, were not seen until later in the incubation period. All of the hydroxylated metabolites were seen during the later stages of the incubation period. These results were similar to the transformation of metabolites measured in the biometers even though the fungal enzyme solution without the fungus was used for herbicide degradation. One difference was that hydroxyatrazine (OEIT) was not found as a metabolite in the EES-treated biometer flasks. This was probably due to the fact that the use of the EES increased the transformation rates of metabolites. An increase in the dechlorinated N-dealkylated metabolite (OAAT) occurred early in the experiment. Since there was an additional subsequent increase in OAAT that coincided with an increase in hydroxyatrazine (OEIT), the initial increase in OAAT suggested that the OAAT formed early in the incubation period was derived from bis-N-dealkylated metabolite (CAAT) rather than OEIT. The low concentration of CAAT compared to CEAT and CAIT was additional evidence of this conclusion. In the work by Mougin et al. [20], both hydroxylated and/or N-dealkylated metabolites were also found supporting the theory that the enzymatic combustion process initiated by the EES produced free radicals that could attach to multiple sites on the AT molecule. The formation of these many compounds during the degradation of AT illustrated the complexity of the EES catalytic process and suggested the existence of many degradation routes that resulted in complex interconnected pathways.

Alachlor degradation resulted in the formation of DMA and DIE, but only DMA was further transformed. The lack of correlation between the amounts of DMA and DIE transformed suggested that other reactions within the soil system were involved in the transformation of DIE. The increase in DMA coincided with a gradual decrease in DIE indicating that transformation of both metabolites occurred simultaneously. The significant difference between the initial DIE concentration in the treated flasks and in the control flasks suggested that degradation occurred very rapidly with

further transformation of DIE as the rate-limiting step. There was a greater amount of both DIE and DMA in the soil degradation study than in the EES-treated aqueous cultures. As with the degradation of AT, the presence of soil components hindered further transformation of the AL metabolites through the formation of bound residues.

5. Conclusions

Treatment of a highly contaminated mix-load site soil with fungal enzymes resulted in the degradation of the herbicides, atrazine, and alachlor. Some mineralization of both herbicides occurred during incubation; however, metabolites of both AT and AL were detected within the treated soil. Degradation of the metabolites also occurred during incubation, indicating that treatment with fungal enzymes can be a viable option in the remediation of contaminated soils.

The presence of other contaminants within the soil did not inhibit AT and AL degradation. In fact, other herbicides present in the soil were also degraded during the study. However, the high concentrations of these other compounds may have reduced the amount of degradation of the target herbicides, AT and AL. Multiple applications of the EES may result in further degradation of all herbicides present in the mix-load site soil. More research on the ability of the EES to react with multiple substrates is warranted.

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